Exhibit A

PERSPECTIVE

Learning the Chemical Language of Cell-Surface Interactions

Jay T. Groves

(Published 13 September 2005)

Living cells have exquisite abilities to sense, respond to, and even remodel their surrounding environment. Cell membranes bristle with receptor proteins, which serve as the primary conduits for transduction of external information into the cell. Innumerable life processes, from the differentiation of embryonic cells into diversified tissues to the formation of memories, are governed by signals from cell surface receptors. In some cases, ligands for receptors are soluble molecules, such as hormones or neurotransmitters. Alternatively, many receptors bind surface ligands, which may be associated with other cells or with extracellular materials.

Surface-bound ligands present an additional layer of complexity in that collective properties of the substrate may influence the response of cells. For example, spatially clustered ligands can elicit a different response from that produced by a uniform distribution of the same average density (*1*). At another level, the mechanical compliance of the substrate material, which dictates how it will deform under forces applied by the cell, is influential. Tensile forces within the cell (*2*), as well as the overall cellular geometry (*3*), can modulate intracellular signaling activity, thus altering the cellular response to external ligands. Such regulatory couplings have been implicated in tissue development, wound healing, and cancer (*4*, *5*).

From a technological perspective, there is tremendous motivation to learn how to design material surfaces that elicit specific cellular responses. Beyond the perpetual need for traditional medical implants, other emerging applications involve the use of substrate materials to coax cultured cells into specific modes of behavior. These can have utility for cell-based screens of pharmaceutical compounds or the development of single-cell–level diagnostic assays. Intriguing nonmedical applications include the stable integration of living cells into devices to allow sensing (*6*, *7*) or even biological energy transduction (*8*). All of this hinges on decoding, and implementing within a synthetic material, components of the chemical language through which cells receive information about the surfaces they contact. This is a complex task, which involves the hierarchical assembly of molecular signaling ligands into materials with appropriate physical properties. Encouragingly, recent experimental breakthroughs illustrate the existence of accessible synthetic design rules.

For example, by adsorbing the extracellular matrix (ECM) protein fibronectin onto surfaces displaying different chemical moieties, García and co-workers were able to modulate its integrin-binding specificity (*9*). The difference proved sufficient to regulate cellular functions; in this case, differentiation and mineralization of osteoblasts (bone-forming cells). What is inspiring about this particular result is that relatively nonspecific chemical modifications of the underlying substrate could be used to toggle a highly specific balance among receptor-ligand interactions. More generally, this observation emerges amid a number of recent advances in the development of chemically tailored synthetic surfaces for cellular interfacing (*10*–*13*). Rapid progress at this junction between materials chemistry and cell biology is expanding our capabilities to engineer communicative interfaces between living and nonliving systems (Fig. 1).

Department of Chemistry, University of California, Berkeley, CA 94720, USA. E-mail, JTGroves@lbl.gov

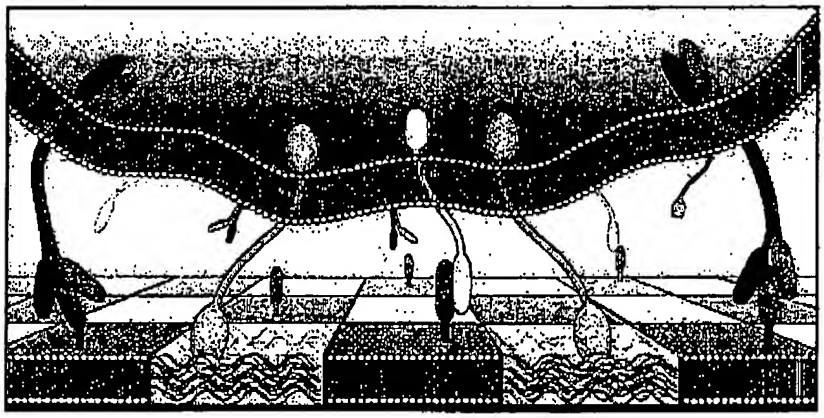

Fig. 1. Schematic of a hybrid interface between a living cell and a synthetic substrate. Receptor proteins on the cell surface engage their corresponding ligands, displayed within a synthetic material, on the underlying substrates. The spatial arrangements of the signaling ligands, as well as their mobility and the local chemical microenvironment, all become integrated into the overall cellular response.

Cell–Extracellular Matrix Interactions

Most mammalian cells receive cues required for normal metabolic activities from the surrounding ECM in vivo (*14*). Correspondingly, efforts to develop synthetic materials to control cell behavior have focused on mimicking or reconstituting the ECM in various ways (*12*, *13*). Natural ECM consists of a fibrous mesh of proteins (including fibronectin, laminin, vitronectin, collagens, and proteoglycans) that serves as both a structural scaffold and a substrate for the display of signaling ligands. Especially prominent among ECM-embedded signaling motifs is the Arg-Gly-Asp (RGD) tripeptide sequence, which is a ligand for roughly a third of the 25 known integrin receptor proteins on cell surfaces (*15*). Integrins are the predominant adhesion receptors responsible for cell-ECM engagement, and also participate in cell-cell interactions. Integrin binding to the RGD sequence is contextual, exhibiting a notable dependence on the RGD microenvironment (*16*). This is, perhaps, unsurprising given the large size of the integrin receptors, though the precise molecular determinants remain unclear. Nonetheless, various strategies of displaying small RGD-containing peptide sequences on synthetic surfaces have been successful in controlling the adhesion and growth of cells (*17*–*20*).

A seemingly more natural way of rebuilding a surrogate ECM on a synthetic substrate might be to directly adsorb whole ECM proteins. However, the results of García and co-workers illustrate how even whole ECM proteins can exhibit environmental sensitivity with respect to their integrin-binding specificities (*9*). Self-assembled monolayers of alkanethiols were used to derivatize gold substrates so that various chemical moieties (OH, NH_2, COOH, and CH_3) were presented on the surface. The response of osteoblast cells to fibronectin, which had been adsorbed onto the surfaces, was then characterized as a function of the terminal surface chemistry. Through a controlled set of experiments, these researchers demonstrated that integrin-binding specificities for the adsorbed fibronectin, and ultimately cellular function, correlate with the underlying surface chemistry. The way in which this occurs is particularly interesting: Two different integrins, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, compete for the RGD binding motif on the fibronectin. Apparently, the underlying surface chemistry alters the fibronectin structure in a manner that shifts the balance between preferential binding of one or the other of these two integrins. These differences, in turn, are transmitted to the cell and modulate osteoblastic gene expression and mineralization, which are related to bone production. Thus, the cell senses the structure of the fibronectin and regulates its own differentiation accordingly. Whether this sort of chemical toggle switch is a common paradigm that can be used in a predictive manner remains to be seen.

A more invasive strategy to engineer cell-surface interactions involves rewiring the cellular adhesion machinery itself. Mrksich and co-workers used genetic manipulations to install a chimeric cell surface receptor with altered ligand specificity (*21*). The engineered receptor contains the intracellular and transmembrane domains of the β_1 integrin. From inside the cell, it resembles the $\alpha_5\beta_1$ integrin mentioned above. However, the extracellular portion of the chimera is composed of a stalk domain (serving as a spacer) and terminates with a binding domain from carbonic anhydrase IV (CAIV). The CAIV domain selectively binds benzenesulfonamide (BzS), providing new specificity for the receptor. Cells expressing this modified receptor adhered and spread selectively on substrates displaying the BzS ligand. The spreading behavior, in particular, required proper signaling from the intracellular integrin β_1 domain, suggesting that the chimera indeed functionally signaled as an integrin receptor. The modularity of this strategy is enticing; it suggests that a wide variety of differing binding specificities could be interchanged with the natural repertoire of intracellular signaling specificities.

Cell-Cell Signaling

Direct signaling between cells may represent an even more diverse problem than that of cell-ECM interactions. In cell-cell signaling, both receptors and ligands can reside in cell membranes, allowing for complex and active feedback from both interacting cells. This feedback can occur at many levels, from increased or decreased protein expression on the cell surfaces to cytoskeleton-driven spatial rearrangements of the receptors and ligands themselves. Neuronal synapses are widely known examples of intercellular signaling junctions. There has been interest in developing synthetic interfaces between neurons and solid-state materials, such as semiconductor integrated electronics. Although there have been successes with electronic triggering and detection of neuronal action potentials (*7*), the prospect of forming a genuine neuronal synapse with a synthetic device remains distant. The crux of the problem is similar to that encountered in cell-ECM interactions: The necessary signaling ligands must be displayed within an appropriate context to direct the cell into the desired mode of behavior.

One strategy of creating synthetic substrates displaying cell surface ligands makes use of the supported lipid membrane architecture (*22*). Lipid bilayer vesicles can spontaneously adsorb and fuse with certain materials, such as silica, to form a single continuous membrane coating on the underlying solid support (Fig. 1). The resulting supported membrane retains the lateral fluidity of the original membrane; proteins can be incorporated and displayed by this technique as well. Supported membranes have proven particularly useful in studies of cell-cell interactions of the immune system (*11*, *23*). Supported membranes have been made that incorporate membrane-linked forms of major histocompatibility complex (MHC) protein; loaded with an appropriate antigenic peptide, along with intercellular adhesion molecule (ICAM). Such supported membranes are sufficient to induce a living T cell to form an immunological synapse with the supported membrane. Striking images of the evolving spatial pattern of the immunological synapse have been obtained by this method (*23*), underscoring the importance of membrane fluidity to allow the large-scale spatial movements of proteins that must occur. At some levels, early stages of synaptogenesis and postsynaptic partner recognition in the central nervous system represent a similar task to that confronted by T cells during surveillance (*24*). Display of neuroligin-1 in supported membranes, representing the postsynaptic cell surface, has also proven sufficient to elicit the onset of synaptogenesis in cultured neurons (*25*). The supported membrane platform provides a promising general strategy for the reassembly of synthetic cell surfaces, while retaining key material properties such as lateral fluidity.

In living tissues, cells generally make contacts with the ECM and with other cells. Spatially juxtaposed signals from different sources combine to direct the full range of cellular behaviors. It is becoming increasingly clear that the arrangement of signaling molecules into large-scale spatial patterns, which

supersede the molecular-scale clustering mentioned earlier, is a key aspect of cell signaling at interfaces (*26*). Simply changing a cell culture substrate from a flat surface to a three-dimensional gel matrix has marked effects on cellular behavior, even eliciting normal growth from cells previously exhibiting cancerous tendencies (*4*). In the case of the T cell, the changing spatial pattern of signaling molecules within the synapse is thought to provide a mechanism to either enhance or attenuate signaling as needed (*27*).

The spatial pattern of signaling molecules presented to cells may be as important as their chemical microenvironment. Fortunately, a wide array of micro- and nanofabrication technologies, which have largely been developed for the semiconductor industry, can be applied to the patterning of proteins and membranes on surfaces (*28–31*). Cells readily react to spatially patterned molecules on synthetic surfaces (*3*, *32*), and applications of such technologies to control and dissect signaling interactions are beginning to appear (*10*, *33*, *34*).

Conclusion

The potential complexity within cellular signaling networks is nearly infinite. At the same time, surprisingly simple changes in the presentation of a signaling ligand can toggle between discrete cellular behaviors. There is a robustness to cellular responses that is tremendously beneficial when employing synthetic materials to control cells. It may not be necessary to exactly reproduce the environment within a living organism to communicate to a cell. Perhaps only certain essential fragments of the cell's natural chemical language are required to trigger a desired response. Learning what these are is key. Recent advances in the development of interfaces between live cells and synthetic materials indicate accelerating progress and are suggestive of significant growth potential at this juncture.

References

1. G. Maheshwari, G. Brown, D. A. Lauffenberger, A. Wells, L. G. Griffith, Cell adhesion and motility depend on nanoscale RGD clustering. *J. Cell Sci.* 113, 1677–1686 (2000).
2. F. J. Alenghat, D. E. Ingber, Mechanotransduction: All signals point to cytoskeleton, matrix, and integrins. *Sci. STKE* 2002, pe6 (2002).
3. C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, D. E. Ingber, Geometric control of cell life and death. *Science* 276, 1425–1428 (1997).
4. M. J. Bissell, A. Rizki, I. S. Mian, Tissue architecture: The ultimate regulator of breast epithelial function. *Curr. Opin. Cell Biol.* 15, 753–762 (2003).
5. D. E. Ingber, Mechanobiology and diseases of mechanotransduction. *Ann. Med.* 35, 564–577 (2003).
6. V. B. Pizziconi, D. L. Page, *Imaging and Characterization of Macromolecular Interface Structures for Whole Cell Biosensors* (Plenum, New York, 1998).
7. R. A. Kaul, N. I. Syed, P. Fromherz, Neuron-semiconductor chip with chemical synapse between identified neurons. *Phys. Rev. Lett.* 92, 038102 (2004).
8. G. Reguera, K. D. McCarthy, T. Mehta, J. S. Nicoll, M. T. Tuominen, D. R. Lovley, Extracellular electron transfer via microbial nanowires. *Nature* 435, 1098–1101 (2005).
9. B. G. Keselowsky, D. M. Collard, A. J. García, Integrin binding specificity regulates biomaterial surface chemistry effects on cell differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5953–5957 (2005).
10. M. Mrksich, What can surface chemistry do for cell biology. *Curr. Opin. Chem. Biol.* 6, 794–797 (2002).
11. J. T. Groves, M. L. Dustin, Supported planar bilayers in studies of immune cell adhesion and communication. *J. Immunol. Methods* 278, 19–32 (2003).
12. J. Hubbell, Materials as morphogenetic guides in tissue engineering. *Curr. Opin. Biot.* 14, 551–558 (2003).
13. R. Langer, D. A. Tirrell, Designing materials for biology and medicine. *Nature* 428, 487–492 (2004).
14. N. Boudreau, M. J. Bissell, Extracellular matrix signaling: Integration of form and function in normal and malignant cells. *Curr. Opin. Cell Biol.* 10, 640–646 (1998).
15. R. O. Hynes, Integrins: Bidirectional, allosteric signaling machines. *Cell* 110, 673–687 (2002).
16. B. T. Houseman, M. Mrksich, The microenvironment of immobilized Arg-Gly-Asp peptides is an important determinant of cell adhesion. *Biomaterials* 22, 943–955 (2001).
17. C. Roberts, C. S. Chen, M. Mrksich, V. Martichonok, D. E. Ingber, G. M. Whitesides, Using mixed self-assembled monolayers presenting RGD and (EG)3OH groups to characterize long-term attachment of bovine capillary endothelial cells to surfaces. *J. Am. Chem. Soc.* 120, 6548–6555 (1998).
18. T. Pakalns, K. L. Haverstick, G. B. Fields, J. B. McCarthy, D. L. Mooradian, M. Tirrell, Cellular recognition of synthetic peptide amphiphiles in self-assembled monolayer films. *Biomaterials* 20, 2265–2279 (1999).
19. A. Rezania, K. E. Healy, Biomimetic peptide surfaces that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteoblast-like cells. *Biotechnol. Prog.* 15, 19–32 (1999).
20. T. W. Jensen, B.-H. Hu, S. M. Delatore, A. S. Garda, P. B. Messersmith, W. M. Miller, Lipopeptides incorporated into supported phospholipid monolayers have high specific activity at low incorporation levels. *J. Am. Chem. Soc.* 126, 15223–15230 (2004).
21. M. Kato, M. Mrksich, Rewiring cell adhesion. *J. Am. Chem. Soc.* 126, 6504–6505 (2004).
22. E. Sackmann, Supported membranes: Scientific and practical applications. *Science* 271, 43–48 (1996).
23. A. Grakoui, S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, M. L. Dustin, The immunological synapse: A molecular machine controlling T cell activation. *Science* 285, 221–227 (1999).
24. M. L. Dustin, D. R. Colman, Neural and immunological synaptic relations. *Science* 298, 785–789 (2002).
25. C. Dean, F. G. Scholl, J. Choih, S. DeMaria, J. Berger, E. Isacoff, P. Scheiffele, Neurexin mediates the assembly of presynaptic terminals. *Nat. Neurol.* 6, 708–716 (2003).
26. J. T. Groves, Molecular organization and signal transduction at intermembrane junctions. *Angew. Chem. Int. Ed. Engl.* 44, 3524–3538 (2005).
27. K.-H. Lee, A. R. Dinner, C. Tu, G. Campi, S. Raychaudhuri, R. Varma, T. N. Sims, W. R. Burack, H. Wu, J. Wang, O. Kanagawa, M. Markiewicz, P. M. Allen, M. L. Dustin, A. K. Chakraborty, A. S. Shaw, The immunological synapse balances T cell receptor signaling and degradation. *Science* 302, 1218–1222 (2003).
28. S. P. A. Fodor, J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu, D. Solas, Light-directed, spatially addressable parallel chemical synthesis. *Science* 251, 767–773 (1991).
29. J. T. Groves, N. Ulman, S. G. Boxer, Micropatterning fluid lipid bilayers on solid supports. *Science* 275, 651–653 (1997).
30. R. S. Kane, S. Takayama, E. Ostuni, D. E. Ingber, G. M. Whitesides, Patterning proteins and cells using soft lithography. *Biomaterials* 20, 2363–2376 (1999).
31. J. T. Groves, S. G. Boxer, Micropattern formation in supported lipid membranes. *Acc. Chem. Res.* 35, 149–157 (2002).
32. R. Singhvi, A. Kumar, G. P. Lopez, G. N. Stephanopoulos, D. I. C. Wang, G. M. Whitesides, Engineering cell shape and function. *Science* 264, 696–698 (1994).
33. Y. Soen, D. S. Chen, D. L. Kraft, M. M. Davis, P. O. Brown, Detection and characterization of cellular immune responses using peptide-MHC microarrays. *PLoS Biol.* 1, e76 (2003).
34. M. Wu, D. Holowka, H. G. Craighead, B. Baird, Visualization of plasma membrane compartmentalization with patterned lipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 13798–13803 (2004).

Citation: J. T. Groves, Learning the chemical language of cell-surface interactions. *Sci. STKE* 2005, pe45 (2005).